(2)

Research Progress Report

submitted to the

U.S. Naval Medical R & D Command

Contract No. N00014-90-C-0053

September 30, 1990

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RESEARCH PROGRESS REPORT

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U.S. NAVAL MEDICAL R&D COMMAND

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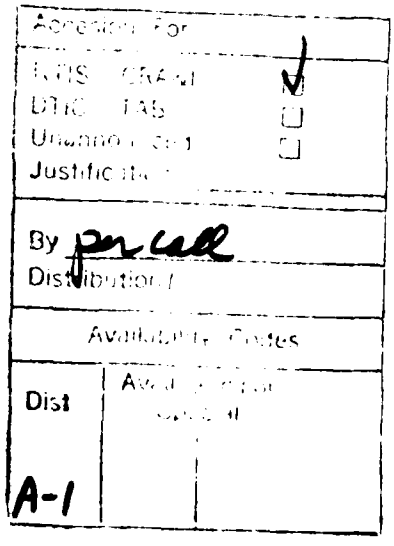

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CRYOPHARM CORPORATION

September 30, 1990

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SUMMARY

The research described in this report has been supported in part by a contract from the United States Navy which began on May 15, 1990. This report includes research conducted at Cryopharm Corporation since September 1989, when we submitted our research proposal that served as the basis for awarding this contract.

Since September 1989 Cryopharm has focused on two primary goals: 1) conduct of the first clinical test of lyophilized reconstituted human red cells in normal volunteers, and 2) continued basic research to characterize and improve the yield and quality of reconstituted red cells. As detailed in this progress report, Cryopharm has met a series of milestones (detailed in the Milestones Chart submitted in our September 1989 proposal and duplicated as Appendix II of this report) designed to position the company to begin human testing of autologous lyophilized red cells. These milestones include definition of a working shelf lyophilization cycle to implement for the first human test samples, construction of a Class 100 clean room facility, validation of sterile processing, and development of a working prototype lyophilization container. Preliminary studies of the residual moisture and shelf life of lyophilized human red cells have also been conducted. We believe that significant progress has been attained towards our proposed Year 1 milestones.

In Appendix I of this report we include a photographic description of the current Cryopharm lyophilization and reconstitution process, to illustrate the blood container, Class 100 facility, and current reconstitution/wash procedure. Our basic research continues to focus on improved techniques to streamline the entire process. For example, ongoing research to characterize the biochemical and physiological properties of lyophilized reconstituted human red cells will help design more rapid rehydration and wash conditions, with an eye toward our Year 3 milestones of streamlining and formal clinical trials.

Our future plans for the next progress period (September 30 through November 9, 1990) include the conduct of initial clinical research with lyophilized human red cells. As discussed under Future Plans in this report, our goal will be to establish a starting baseline for in vivo survival of autologous, small doses. This starting baseline will provide the first indication of how the in vitro properties of lyophilized human red cells (including cell metabolism, membrane/cytoskeleton structure, hemoglobin preservation, and deformability) characterized and discussed in this report correlate with in vivo circulation. The direction and extent of further improvements to lyophilized red cells are predicated on these initial cell survival evaluations.

RESEARCH REPORT

I. Optimizing of Red Cell Lyophilization and Reconstitution.

Since the submission of Cryopharm's research proposal in September 1989, we have focused on our first year goals of developing a working sterile blood container for lyophilization, and integrating this container with an efficient lyophilization cycle and subsequent handling during reconstitution. As shown in the photographic series in Appendix I of this report, Cryopharm's first container design has proven effective in allowing sterile drying and rehydration of human red cells. The container can be placed on the shelf of a standard pharmaceutical freeze-dryer, and has sterile docking ports compatible with existing blood bag connectors. We envision that this general-purpose container design will meet the needs of our early clinical studies, and that similar containers can be used for future lyophilized products, such as human platelets.

Sterile Container Design and Testing.

As shown in Appendix I, fresh human packed red cells received from volunteers is processed in a Class 100 clean room facility containing two research-scale shelf lyophilizers. The thermoformed PVC-DEHP bag is manufactured from materials compatible with biologic products and is sterilized by ethylene oxide exposure by the manufacturer. Containers are certified as sterile, pyrogen-free, and at FDA required levels of ethylene oxide residuals. The configuration allows a single unit of packed red cells (about 200-250 ml of cells) to be lyophilized as a homogeneous layer. Cryopharm is currently studying various particle barrier designs to be incorporated in the central vapor-removal port, to preclude cross-contamination between units. For the initial human studies, only one autologous unit will be processed in each lyophilizer per cycle.

Mock drying studies using sanitized lyophilizers, the Class 100 clean room, and EtO-treated bags have shown that full units of cells can be dried and rehydrated in the primary bag, and then processed in a Cobe cell washer without introduction of microbial contaminants. These studies have addressed the general safety issues required for low-dose human trials.

Lyophilization Cycle Testing.

Cryopharm has developed a working lyophilization cycle, using shelf model freeze-dryers, that reliably dries full red cell units to at least 3% residual moisture (see Section III for residual moisture data). Our current cycle is tailored to the existing lyophilization container design and current protective solutions. For the planned initial clinical research, an extensive 10 day drying cycle will be used to ensure complete drying and no sample collapse (see Appendix I for a photograph of uniformly dried red cells). Subsequent scale-up goals will involve reducing the cycle time to 5-7 days to achieve more efficient production cycle lengths. These scale-up goals will be addressed once the fundamental issues of buffer formulation and cell survival baseline determination are concluded.

Reconstitution Design.

Appendix I also illustrates our current reconstitution process. Fluid is introduced

into the lyophilization container via a sterile port, and shaken on a wrist-action shaker for 30 minutes. Our next goal is to reduce the time needed for thorough rehydration of the dried cell layer. Incorporation of mixing baffles inside the thermoform bag, or ultimately using pelleted freeze-dried compositions, may accelerate rehydration. This issue will be addressed once adequate cell recovery and in vivo survival are demonstrated.

II. In vitro Properties of Reconstituted Human Red Cells.

Adenine Nucleoside Synthesis Measured by 14-C Adenine Incorporation

In our September 1989 research proposal we reported that lyophilized reconstituted human red cells retained a functional glycolytic pathway as measured by lactate production in vitro. The ability of stored red blood cells to maintain ATP levels has been correlated with their survival in vivo (1). Synthesis of ATP is also dependent upon uptake of adenine, which is used as a precursor for maintenance of the cellular pool of adenine nucleosides. We have extended our studies of lactate production in lyophilized reconstituted red cells, which provide an end-product measure of glycolytic function, to include a direct measurement of the rate of uptake of a key ATP precursor into lyophilized reconstituted red cells.

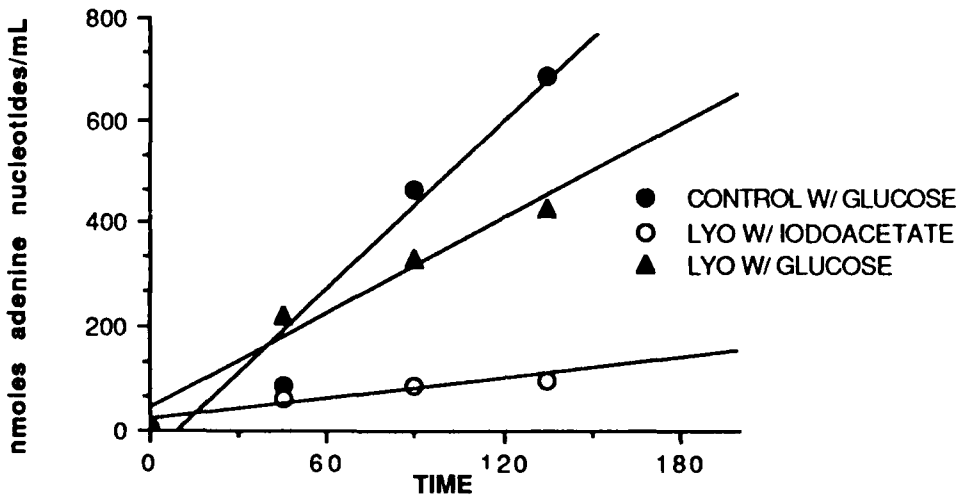
In collaboration with Dr. Kouichi Tanaka of the Harbor UCLA Hospital, we have measured the rate of uptake of 14-C labeled adenine into ethanol-precipitable adenine nucleosides. The 14-C adenine incorporation method has been described (2), as well as the method used to separate free 14-C adenine from newly made 14-C adenine nucleosides (3). As shown in Graph 1, we have compared the rate of incorporation of free labeled adenine into the nucleoside pool in fresh human red cells versus lyophilized reconstituted red cells. Glucose is added to the assay mixture to drive glycolysis. As a further control, we also incubated lyophilized reconstituted red cells in the same assay mixture containing iodoacetate, a potent metabolic inhibitor. In this graph each point represents the average value from two independent experiments. From the slopes of the plotted lines the relative rates of incorporation can be determined, and are listed below.

Table 1: Calculated Rates of 14-C Adenine Incorporation into Nucleoside.

Experimental Condition	Rate of Incorporation (nmoles adenine nucleoside/ml RBC x min.)
Fresh RBC Control	5.36
Lyophilized RBC	3.06
Lyophilized RBC with iodoacetate added	0.66

These data show that lyophilized reconstituted human red cells retain about 60% of the rate of 14-C adenine incorporation into precipitable nucleoside exhibited by fresh

GRAPH 1
14C-ADENINE INCORPORATION



control cells. The ability of lyophilized reconstituted cells to incorporate the precursor can be poisoned by addition of iodoacetate, an inhibitor of many enzymes. These data support our prior lactate synthesis and steady-state ATP level studies of lyophilized human red cells, and suggest that the metabolic machinery is reasonably intact. The ability of lyophilized to maintain ATP synthesis de novo from free precursor after reconstitution may contribute to the overall in vivo viability of the cells.

Immunologic Crossmatching

The formal possibility exists that red cell surface antigens can be altered, or new antigens can be created, as a consequence of the lyophilization and reconstitution procedure. Such hypothetical alterations could cause recipients to become sensitized to lyophilized cells. As a first step in addressing this issue, we have conducted extensive compatibility testing using both autologous and group specific lyophilized crossmatches.

In these studies both polyspecific (anti-IgG, C3d) and monospecific (anti-IgG) anti-human globulins were used to test lyophilized reconstituted red cells for incompatibility as measured by the standard agglutination assay. Red cells returned to autologous plasma were then washed and incubated at 37 degrees for 15, 20, 30, 45, and 60 minutes with anti-human globulin. As no differences were observed with incubation time, a standard 30 minute assay was adopted. In addition, control non-group specific crossmatches were conducted to demonstrate the validity of the antiglobulin reagent.

The results of these tests are summarized in Table 2. All lyophilized red cell crossmatches to autologous plasma have yielded compatible results (i.e., no detectable agglutination). Two units that tested non-compatible prior to lyophilization gave the same result after lyophilization (these units are designated by asterisks in Table 2). The same result (no agglutination) has been obtained with 63 group specific lyophilized crossmatches. Nine controls with non-group specific crossmatching to lyophilized cells yielded agglutination, as expected.

These data indicate that lyophilized reconstituted human red cells do not cross-react with antibodies normally present in either autologous or group specific (ABO/Rh(D)) plasma. These results indicate that small, single doses of lyophilized red cells should not be associated with disseminated intravascular coagulation upon infusion into properly matched recipients, or if given autologously. We have not found any indication that lyophilized cells will react with immune globulins in matched individuals.

These data do not preclude the possibility that large doses, or multiple doses may sensitize certain recipients, in spite of proper crossmatching. This formal possibility will be addressed during the appropriate phase of clinical testing, once the basic clinical research issues of cell survival and low dose safety have been addressed.

TEM Examination of Erythrocyte Ghosts

In collaboration with Drs. Jiri Palek and David Liu of Tufts University School of Medicine, Cryopharm has conducted a transmission electron microscopic (TEM) examination of red blood cell ghosts prepared from fresh control cells and lyophilized reconstituted cells. This technique permits direct visual examination of the cytoskeleton

TABLE 2

Autologous Crossmatch

Group/Rh$_o$(D)	Polyspecific Anti-Human Globulin		Monospecific Anti-Human Globulin	
	compatible	incompatible	compatible	incompatible
Apos	10	1*	7	1*
Aneg	7	1*	0	1*
Bpos	20	0	2	0
Bneg	0	0	0	0
ABpos	24	0	4	0
ABneg	1	0	1	0
Opos	5	0	2	0
Oneg	2	0	0	0
Total	69	2	16	2

Group Specific Crossmatch

Type Specific [ABO/Rh$_o$(D)]

Group/Rh$_o$(D)	Polyspecific Anti-Human Globulin		Monospecific Anti-Human Globulin	
	compatible	incompatible	compatible	incompatible
Apos	20	0	20	0
Aneg	0	0	2	0
Bpos	4	0	4	0
Total	24	0	26	0

Group Specific [ABO/Rh$_o$(D)+/-]

Group/Rh$_o$(D)	Polyspecific Anti-Human Globulin		Monospecific Anti-Human Globulin	
	compatible	incompatible	compatible	incompatible
Aneg > Apos	0	0	3	0
Bpos > Bneg	2	0	2	0
ABps > ABng	3	0	3	0
Total	5	0	8	0

Non-group Specific Crossmatch

Group/Rh$_o$(D)	Polyspecific Anti-Human Globulin		Monospecific Anti-Human Globulin	
	compatible	incompatible	compatible	incompatible
ABng > Apos	0	4	nd	nd
ABng > Aneg	0	2	nd	nd
Total	0	6		

* - Non-lyophilized cells compatibility testing - incompatible, both units were direct Coombs positive.

nd - not determined.

and plasma membrane, and can be used to detect gross aberrations in morphology. These studies indicate that the cytoskeletal protein network appears normal in lyophilized cells compared to fresh control cells (photographic prints are available upon request). This observation is further supported by studies of the gel electrophoretic protein pattern of fresh cells versus lyophilized cells (data not shown), indicating no unusual shifts in electrophoretic mobility by individual cytoskeletal protein species.

The TEM photographs do reveal a more frequent occurrence of apparent membrane lesions in lyophilized cells relative to control cells. Although these same lesions occur in fresh cells, they appear to be more common in lyophilized cells. We suspect that the lesions represent areas of disruption of bilayer integrity. This hypothesis is supported by osmotic stability measurements using NaCl and solutions of large, impermeant osmolytes (data not shown). This work indicates that lyophilized reconstituted human red cells are more permeable to small ions such as NaCl, but not larger osmolytes such as sucrose or dextran.

As will be discussed in the following section on Hemoglobin Preservation, we hypothesize that the membrane lesions result from general oxidative damage to the red cells induced during processing. Now that significant yields ($\geq$ 35%) of intact red cells carrying mostly oxyhemoglobin can be recovered after lyophilization, our future basic research direction will focus on fine-tuning our procedure to improve the yield and cell quality. Minimizing oxidative damage will likely guide our efforts. Establishing a baseline in vivo survival level for lyophilized cells will permit future modifications to be evaluated from an in vitro and in vivo perspective.

Hemoglobin Preservation

1) Levels of Key Hemoglobin Species in Lyophilized Red Cells.

The amount of functional hemoglobin contained in reconstituted lyophilized red cells is a useful indicator of their potential oxygen-transport ability. As a first assay, we have used the spectrophotometric method described by Szebeni (4), which relies on measurements taken at four different wavelengths to determine the levels of oxyhemoglobin, methemoglobin, and hemichrome (the fourth measurement at 700 nm corrects for background scatter). As shown in the Table below, lyophilized reconstituted human red cells retain a high percentage of functional oxyhemoglobin, as detected by this assay.

Table 3: Percentage of Hemoglobin Species in Reconstituted Human Red Cells.

Hemoglobin Species	Assay Wavelength	Percent
Oxyhemoglobin	560 nm	96.6 $\pm$ 2.0
Methemoglobin	577 nm	2.9 $\pm$ 1.9
Hemichromes	630 nm	Not detectable

N = 63

Human red blood cells contain a methemoglobin reductase system to convert methemoglobin to oxyhemoglobin (5). The low levels of methemoglobin detected in lyophilized reconstituted red cells should be reversible in the reconstituted cells. Indeed, enzymatic assays conducted in collaboration with Dr. Kouchi Tanaka indicate that Cryopharm's reconstituted human red cells retain 50-75% of the normal methemoglobin reductase activity.

2) ESR Measurements of Hemin Levels in Lyophilized Red Cells.

Production of the various species of hemichromes can lead to irreversible degradation to hemin (reviewed in 6). Hemin is thought to act like a detergent to disrupt membrane properties (7). In order to directly assess the effects of lyophilization on red cell hemin content, a more sensitive electron spin resonance (ESR) analysis has been conducted in collaboration with Dr. Matthew Platz of The Ohio State University. Our results are shown in the following Table:

Table 4: ESR Measurements of Hemin in Lyophilized Human Red Cells.

Method of Preparation	N	Hemin Concentration (mM)	Lyo/Control
Non-lyophilized Controls:			
Washed	5	0.154 ± 0.152	
Unwashed	4	0.479 ± 0.152	
Lyophilized:			
Non-shifted	3	0.286 ± 0.152	1.8
IHP shifted	5	0.727 ± 0.152	4.7

These data show that refrigerated CPDA red cells (controls) do accumulate hemin during storage, which can be removed by washing. Lyophilized reconstituted cells are washed after reconstitution and contain 1.8 times the hemin of stored, washed red cells. Right-shifting agents such as inositol hexaphosphate (IHP) promote further hemin production, to almost 5 times the level of washed packed red cells. We propose to investigate the use of antioxidants during the preparation of lyophilized reconstituted red cells, to try to minimize the production of potentially harmful hemoglobin degradation products.

The studies of hemin production suggest that general oxidative damage to red blood cells during pretreatment, lyophilization, or reconstitution and washing may contribute to reduced in vitro properties such as cell deformability. As indicated in the preceding section, transmission electron microscopy of membrane ghosts prepared from lyophilized human red cells suggest that disruption of bilayer integrity can occur. The initial damage may involve oxidative damage during processing, which cascades into hemoglobin

degradation and production of undesirable hemin.

3) Determination of P-50 Values in Lyophilized Red Cells.

In the preceding section on hemoglobin preservation, we showed that lyophilized human red cells contain a high percentage (>96%) of oxyhemoglobin. This implies that the reconstituted cells are capable of effectively delivering oxygen to tissues. To extend the initial spectrophotometric measurements, we have measured the in vitro oxygen dissociation properties of lyophilized human red cells, relative to liquid stored cells. The data are summarized below in terms of the P-50 value, or partial pressure of oxygen at which 50% of bound oxygen is dissociated (Hemox analyzer data).

Table 5: P-50 Determination for Stored and Lyophilized Human Red Cells.

CPDA Stored Age (Days)	N	P-50 (mm Hg)
0-7	1	27
19 ± 3	12	23.4 ± 4.3
36 ± 8	8	15.9 ± 2.1
Lyophilized		
Non-shifted *	8	15.4 ± 1.3
IHP shifted +	12	26.3 ± 4.7

* Lyophilized reconstituted cells had been stored in CPDA 27 ± 6 days prior to actual freeze-drying.

+ Lyophilized reconstituted cells treated with IHP during washing had been stored in CPDA 15 ± 3 days prior to actual freeze-drying.

These P-50 measurements indicate that packed red cells stored refrigerated in CPDA do exhibit a decreased oxygen delivery capability upon prolonged storage. This storage effect has been documented and correlates with decreased 2,3-DPG levels in stored red cells (8). Our lyophilized reconstituted red cells exhibit a P-50 value comparable to aged red cells. Treatment with IHP can significantly improve oxygen affinity, but this treatment correlates with increased hemin levels, which we believe are diagnostic of general intracellular oxidative damage (data discussed previously). One future goal of our clinical research is to determine the trade-off between use of right-shifting agents such as IHP and the increased levels of hemin, as they affect in vivo circulation.

Cell Deformability Measured by Ektacytometry

The ektacytometer developed by Dr. N. Mohandas at the University of California, Berkeley, has been used to correlate cell deformability under shear stress with in vivo survival (9, 10). Cryopharm has recently set-up its own ektacytometer in-house to facilitate these studies. Our data are reported as the mean Deformability Index at a relative shear rate (D.I. 200) in 290 mOsmol buffer. In collaboration with Dr. Mohandas, we have studied fresh human red cells versus lyophilized reconstituted cells. This technique provides a convenient first assay for the quality of cells produced using new buffer formulations, or using different lyophilization or washing conditions. The following Table illustrates progress towards defining an optimal overall process, as measured by the mean D.I. 200 value.

Table 6. Ektacytometry Measurements on Control and Lyophilized Human RBCs.

Sample	Preparation Conditions *	Mean D.I. 200	N	% of Control
Control	Fresh, non-lyophilized	0.56 ± 0.04	23	
1	Buffer A, vacuum dried, table-top centrifuge	0.20 ± 0.04	4	36.5 ± 7.6
2	Buffer B, vacuum dried, table-top centrifuge	0.26 ± 0.05	3	46.8 ± 8.5
3	Buffer B, vacuum dried, Cobe processed	0.29 ± 0.01	3	52.2 ± 1.4
4	Buffer C, vacuum dried, table-top centrifuge	0.23 ± 0.01	4	41.6 ± 2.0
5	Buffer C, shelf dried, Cobe processed	0.35 ± 0.03	3	61.7 ± 4.6

(*) Preparation conditions affect the quality of the recovered cells, as illustrated by these data. In this table we distinguish between samples dried in an uncontrolled bench model vacuum dryer versus samples dried in a controlled shelf model lyophilizer (i.e., vacuum drying versus true lyophilization). We also investigate differences between reconstituted samples washed manually with a table-top centrifuge versus automated washing in a Cobe 2991 cell washer.

The data presented in the above Table indicate improvement in the quality of lyophilized reconstituted cells relative to fresh red cell controls. Since the D.I. 200 value has been correlated with cell survival for normal and spherocytic or other abnormal red cells (10), we believe that improvements in D.I. index will correlate with improved

survival. This approach has allowed us to refine not only our cryoprotective buffer solutions (compare buffers A, B, C), but to also examine details of processing, including centrifugation conditions for post-rehydration washes and controlled versus uncontrolled drying.

The data reported above for Buffer C represents the current cryoprotective formulation that we plan to use in our initial clinical research trials. Our goal is to obtain a baseline cell survival measurement to determine conclusively whether our buffer formulation strategy is producing higher quality cells (as measured by in vivo survival).

Sterility of Processed Samples

Cryopharm has conducted a series of trial production runs using its Class 100 clean room facility to lyophilize units of packed human red cells. These trials were designed to test the integrity of ethylene oxide sterilized lyophilization containers, and to test our ability to lyophilize and recover sterile, reconstituted red cells.

Each test involved units of packed human red cells collected at various blood banks and air shipped to Cryopharm. Packed cells were lyophilized using sanitized freeze-dryers in EtO-sterilized bags. Upon reconstitution and washing with sterile buffer solutions prepared in the Class 100 facility, samples of the recovered cells were cultured for aerobic and anaerobic growth using fluid thioglycollate broth (11). For each test 10 ml of reconstituted cells were injected into 100 ml of certified thioglycollate broth, incubated for 72 hours at 30-32 degrees C, and then subcultured into a fresh bottle of thioglycollate. Incubation of both the original culture and the subculture then continued at 30-32 degrees C for at least 7 days. Cultures were inspected daily for turbidity to assess microbial growth.

Sterility testing of units of human red cells lyophilized in Cryopharm's Class 100 facility began in May 1990. As of early September 1990, a total of 34 units have been lyophilized and sealed under Class 100 conditions, then rehydrated and washed using sterile docking connectors to introduce rehydration solutions, and the rehydrated cells removed and cultured in thioglycollate broth. The results of these tests are shown in Table 7.

Table 7. Sterility Testing of Lyophilized Human Red Cells.

No. of Units Tested	No. Sterile	No. Non-Sterile
34	29	5 (*)

(*) Reasons for Non-Sterile Outcome:

Some of the early prototype bags ruptured during processing or exhibited some leakage. Rupture of Cobe processing bags also occurred. All five non-sterile samples were associated with container integrity problems during processing.

III. Storage Properties of Lyophilized Human Red Cells.

Preliminary studies have been conducted to examine critical storage properties of lyophilized human red cells. These studies are preliminary in nature since the final packaging of dried red cells remains to be determined. However, studies of the residual moisture levels of dried red cells achieved with our current drying protocol and studies of shelf life during room temperature or refrigerated storage can be examined using analytical samples dried in glass vials.

Residual Moisture Determination

The level of residual moisture is critical to the shelf life of lyophilized products. Under our current lyophilization conditions, human red cells can be consistently dried to a residual moisture content of about 2%, as shown by three sample lots assayed using the Karl Fischer method of analysis (12).

Table 8: Analysis of Lyophilized Human Red Cells for Residual Moisture.

Sample Lot No.	N	Residual Moisture by Karl Fischer Method
E53793	6	2.02 ± 0.7
E52911	5	2.04 ± 0.6
T13794	18	2.00 ± 0.8

Standard pharmaceutical freeze-drying can achieve residual moisture ranges of 1-3%. We plan to continue to evaluate and improve our lyophilization cycle to achieve effective drying using the shortest cycle time.

Shelf Life Studies at Room Temperature and Refrigerated

To assess the effects of lyophilized storage at room temperature versus refrigerated, 6 ml samples of human red cells were lyophilized in 50 ml vials and stoppered under vacuum upon completion of the drying cycle. All samples were scored visually for dryness and the starting sample residual moisture was in the 2% range that we consistently achieve during shelf drying. Four samples were immediately reconstituted and analyzed to provide a time zero reference point, and the remaining vials were stored in the dark at either room temperature or refrigerated. After 1, 2, 4, 6, 8, and 17 weeks of storage, three vials maintained at each temperature were reconstituted and the recovered cells were pooled before analysis. The percent cell recovery, ATP level, hemoglobin content, and red cell indices were then examined.

As shown in Graphs 2-5, storage at either temperature for up to 17 weeks does not produce significant changes in the red cell indices (MCHC, MCV, MCH), or percent recovery of cellular hemoglobin, relative to the time zero reference samples. A significant

difference is observed between room temperature and refrigerated storage in the level of cellular methemoglobin, as shown in Graphs 6 and 7. In this experiment the hemoglobin findings can be summarized as follows:

Table 9: Effect of Room Temperature or Refrigerated Storage on Hemoglobin Oxidation in Lyophilized Human Red Cells.

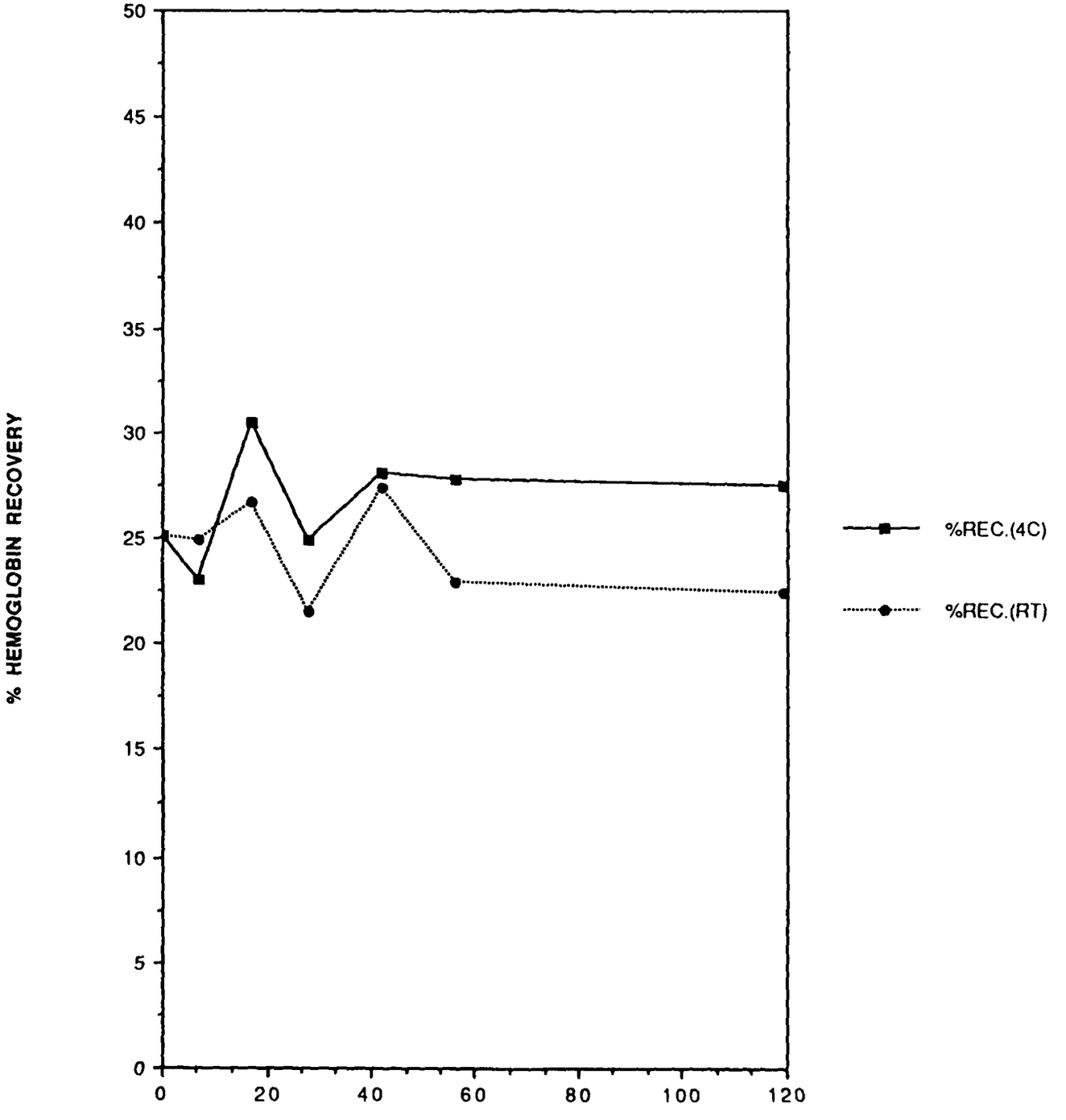
Week	Stored Lyophilized Sample	% Hemoglobin Species Present		
		Oxy Hb	Met Hb	Hemichrome
0	Time Zero (n=4)	92.4	7.6	0
1	Room Temp. (n=3)	90.7	8.2	1.05
	Refrig. (n=3)	98.7	0	1.31
2	Room Temp. (n=3)	88.7	11.4	0
	Refrig. (n=3)	97.1	2.9	0
4	Room Temp. (n=3)	75.6	24.4	0
	Refrig. (n=3)	95.2	4.8	0
6	Room Temp. (n=3)	86.7	13.3	0
	Refrig. (n=3)	99.7	0.34	0
8	Room Temp. (n=3)	82.8	17.2	0
	Refrig. (n=3)	94.5	5.5	0
17	Room Temp. (n=3)	80.6	19.4	0
	Refrig. (n=3)	98.6	1.4	0

The level of methemoglobin increases significantly at room temperature, while refrigerated storage inhibits methemoglobin formation. Although room temperature storage does accelerate methemoglobin production under these conditions, additional studies are needed to determine whether use of antioxidants during processing will retard hemoglobin oxidation during storage (see Future Plans).

In experiments conducted in collaboration with Dr. K. Tanaka of the Harbor-UCLA Hospital, we have found that lyophilized reconstituted human red cells retain the endogenous methemoglobin reductase system (5). Our data indicate that reconstituted cells retain about 50-75% of the enzymatic activity present in fresh control cells. These data suggest that reconstituted cells will be capable of repairing some of the methemoglobin produced during prolonged storage. Further in vitro storage studies, including accelerated decay studies at higher temperatures, coupled with in vivo cell survival studies, will be needed to assess the optimum shelf life at various storage temperatures. These studies can be initiated once the fundamental lyophilization buffer, lyophilization/storage container, and post-rehydration processing issues have been resolved.

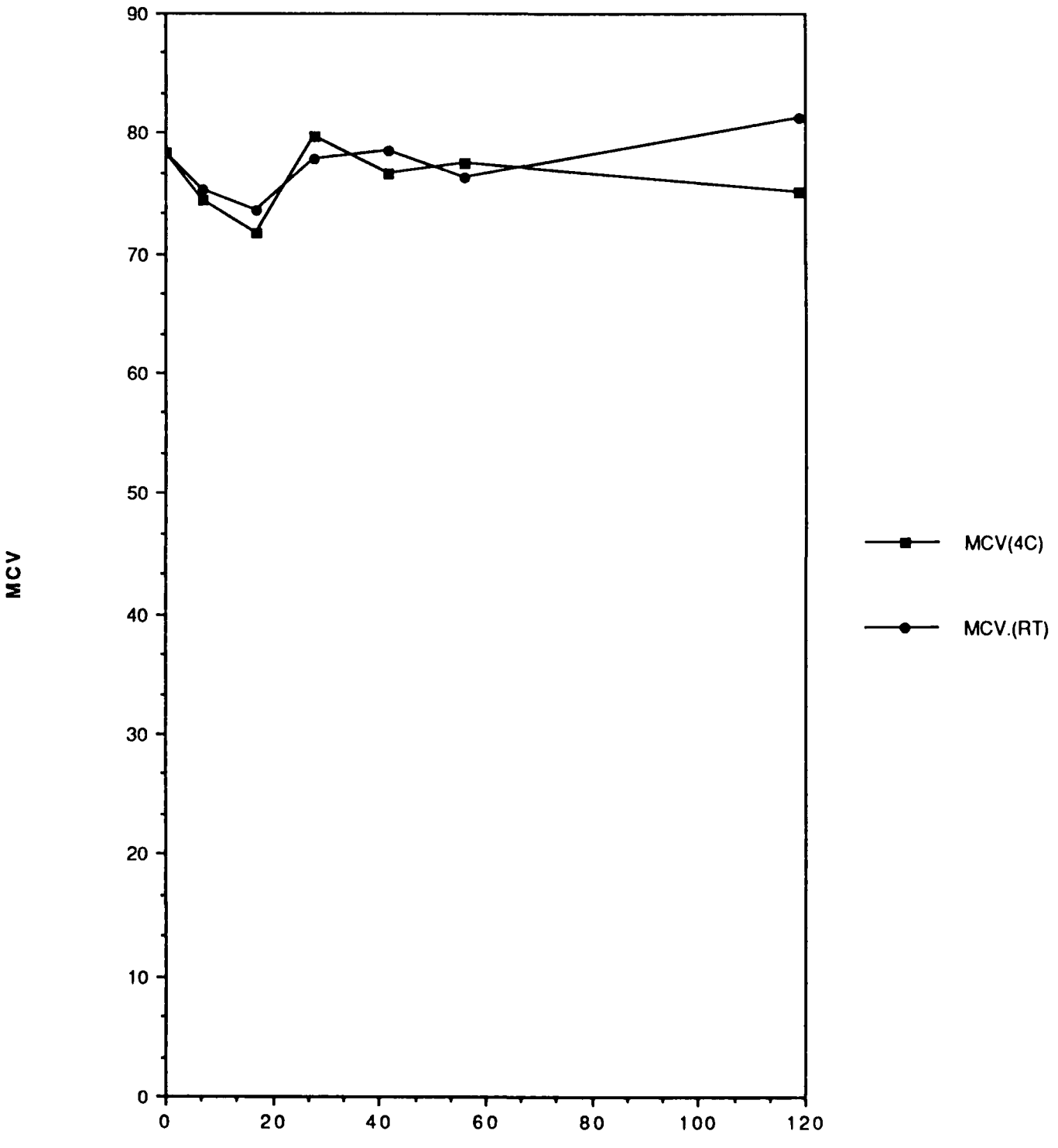
GRAPH 2

HEMOGLOBIN RECOVERY AS A FUNCTION OF STORAGE TIME



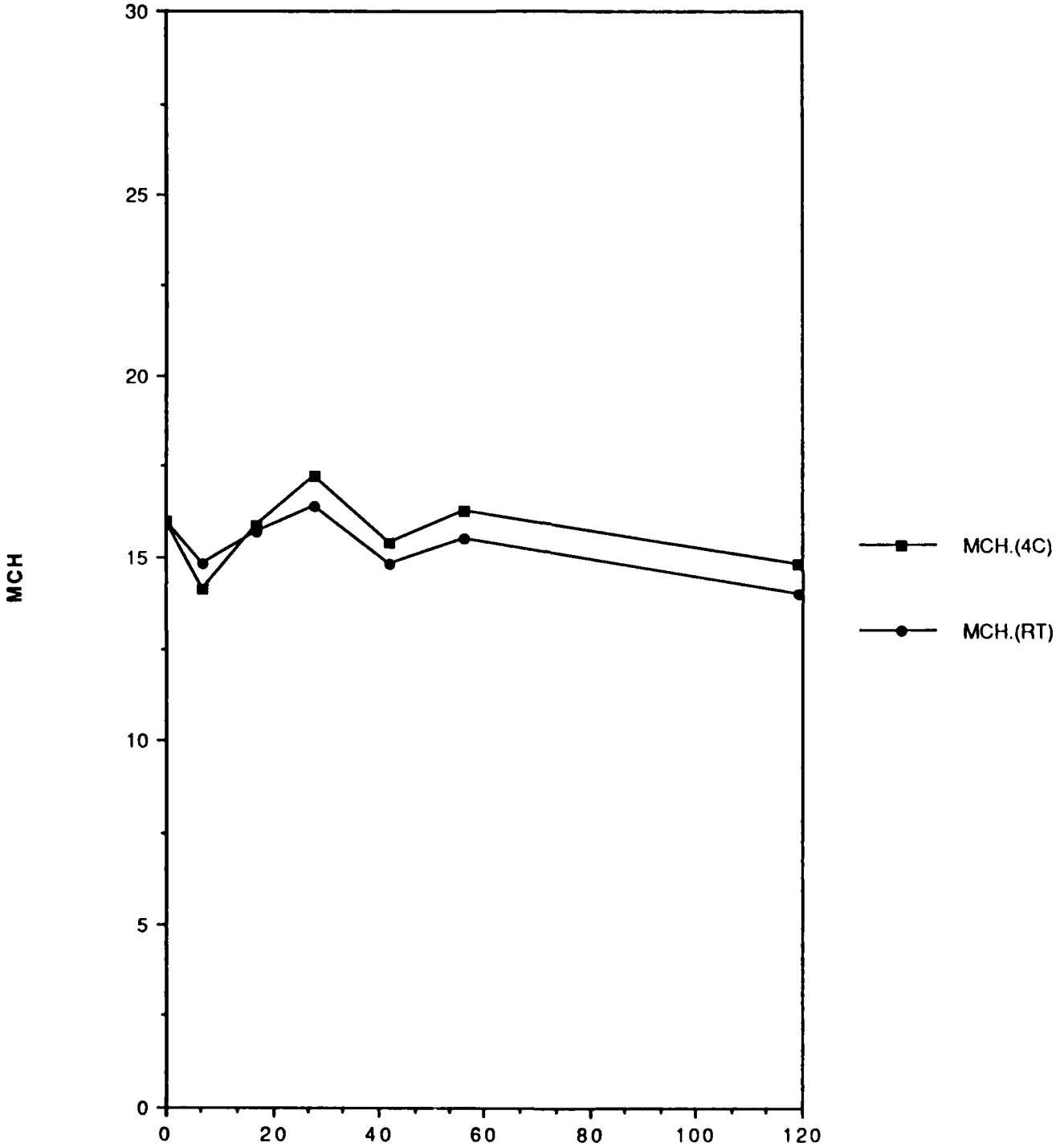
GRAPH 3

MCV AS A FUNCTION OF STORAGE TIME



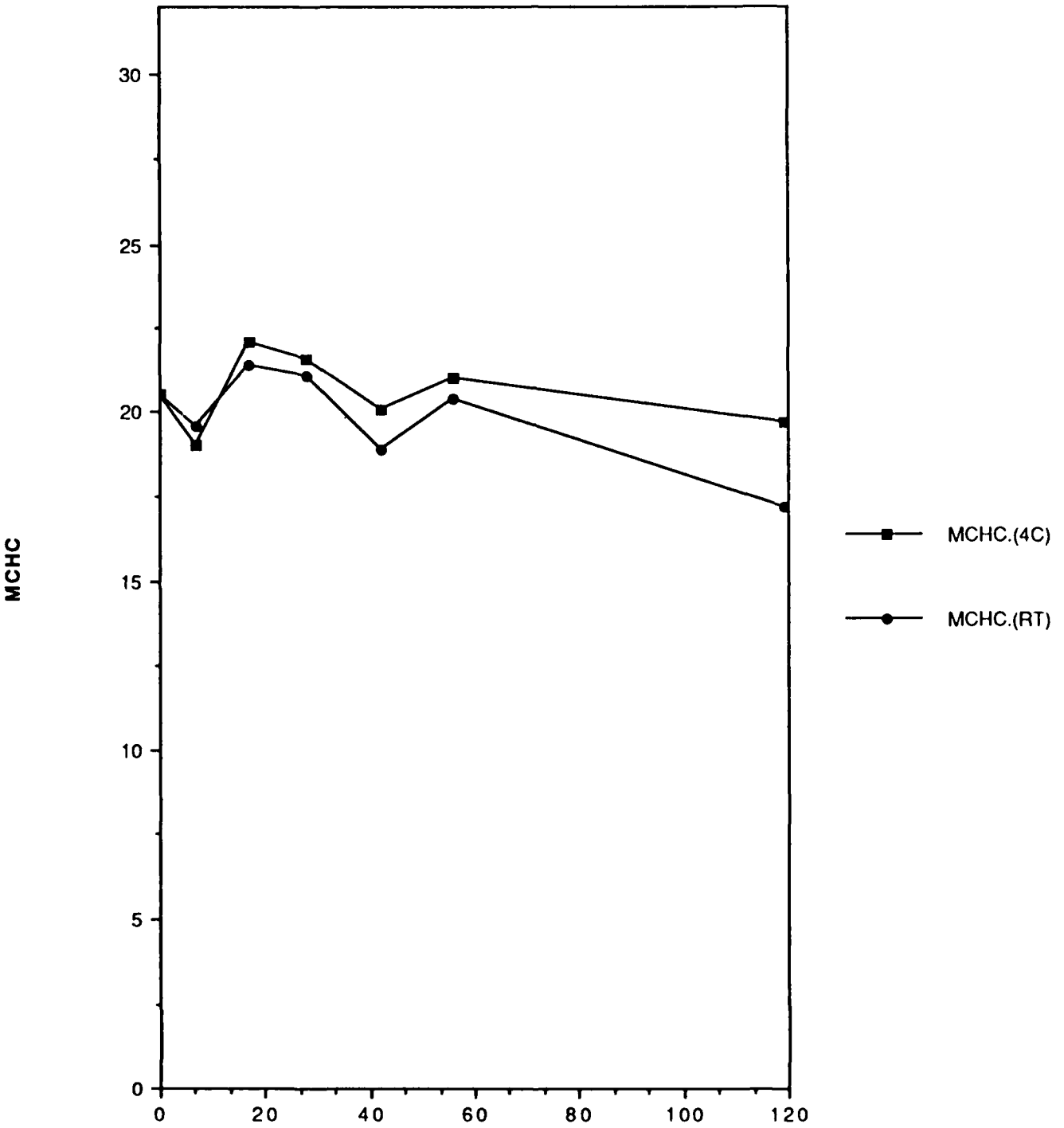
GRAPH 4

MCH AS A FUNCTION OF STORAGE TIME



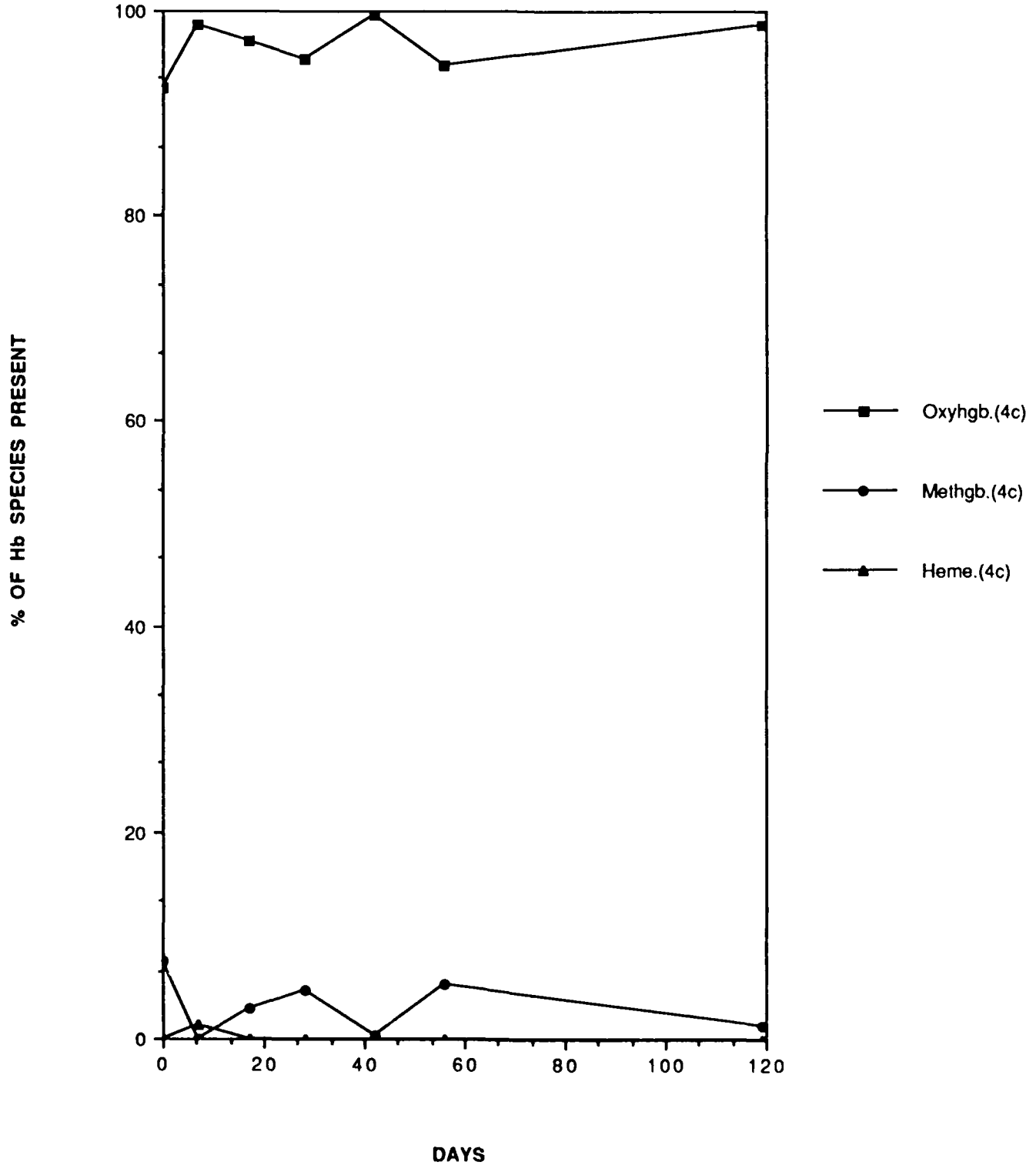
GRAPH 5

MCHC AS A FUNCTION OF STORAGE TIME



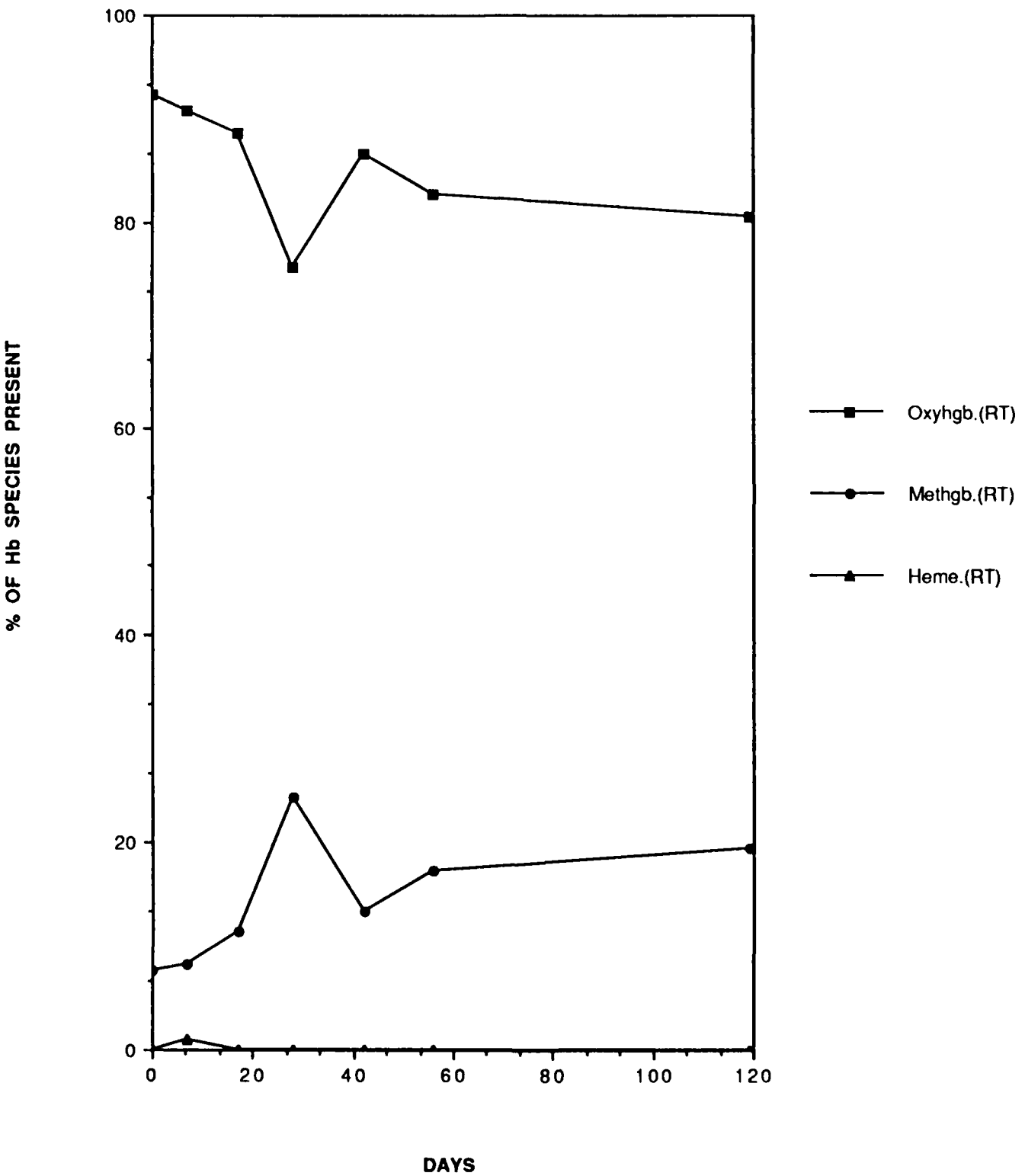
GRAPH 6

% OF Hb SPECIES AS A FUNCTION OF STORAGE TIME AT +4 CELSIUS



GRAPH 7

% OF Hb SPECIES AS A FUNCTION OF STORAGE TIME AT ROOM TEMPERATURE



IV. Evaluation of Animal Red Cells Following Lyophilization.

In Cryopharm's research grant proposal dated September 1989, we discussed attempts to apply our lyophilization buffers, developed with human red cells, to red cells from a number of standard laboratory animal species (see Table 2 in the proposal). Our goal was to assess whether an autologous animal circulation model could be used to evaluate viability of lyophilized reconstituted cells in vivo. We reported in September 1989 that attempts to lyophilize red cells from rats, guinea pigs, dogs, and cynomolgous macaques failed due to apparent species differences in the cells. These results are summarized in Table 10.

Table 10. Summary of Animal Red Cell Lyophilization Studies.

Species	Observed Effects of Lyophilization
Mouse	No cellular recovery. Formation of precipitated Hb and membranes at reconstitution.
Rat	No cellular recovery.
Guinea pig	No cellular recovery.
Dog	Recovery of cells with 80-90% methemoglobin. Only 1/3 as many cells recovered versus human cells.
Pig	Spectrin content 20-30% reduced in reconstituted cells. Deformability reduced to 20% of normal.
Cynomolgus, Rhesus, & Baboon	No detectable levels of glycolytic intermediates. Recovery 2/3 that seen with human cells. No lactate or ATP synthesis. Deformability reduced to 10% of normal. Cynomolgus cells tested incompatible to autologous plasma after lyophilization and reconstitution.
Chimpanzee	Preliminary tests comparable to human values for cell recovery, cell morphology, and ATP and lactate synthesis.

Due to the superior in vitro properties of lyophilized human red cells, Cryopharm plans to conduct low dose, autologous cell survival studies in humans, to directly address the circulation issue. These first studies should provide a baseline value for survival in vivo, free of the added complications introduced by unexplained effects on animal red cells. In addition, in the next section we discuss results of heterologous circulation studies using human cells in tolerant rats. In the absence of a simple autologous model, we have used the heterologous system to obtain a subjective estimate of in vivo viability. Use of a heterologous assay can at least be used to assess whether any in vivo survival can be expected.

V. In vivo Study of 51-Cr Human Red Cells in a Tolerant Rat Model.

One approach used to estimate the in vivo survival of human red blood cells in a heterologous system involves the use of rats rendered tolerant to human cells by treatment with ethyl palmitate and cobra venom factor (13, 14). This method has been used to study survival of labeled human sickle cells and stomatocytes versus control cells from normal subjects (14, 15). In the absence of a viable homologous animal circulation model, we reasoned that this approach will provide a baseline estimate for evaluation of potential process improvements.

The tolerant rat system developed by Castro (14) relies on two reagents to block the reticuloendothelial and complement systems, thereby creating a time window during which the treated rats tolerate infused foreign red cells. Although this compromised, heterologous model is less preferable to autologous animal studies, we believed a useful subjective evaluation of in vivo survival could be obtained, relative to normal controls. This model provides a means of evaluating how well cells are capable of surviving stresses experienced during passage through the capillary system, without considering more rapid destruction due to immune recognition. As such, it serves as an in vivo filtration assay under physiological conditions.

Male Sprague Dawley rats received surgically implanted catheters into the jugular vein. Each rat received an intravenous administration of ethyl palmitate (2 gm/kg body weight) and cobra venom factor (50 anticomplement units/kg). Following pretreatment, 51-Cr labeled human red cells were infused. The 51-Cr labeling efficiency was comparable for all samples (see data in Table 11, which are values from five studies). Animals received fresh human red cells, heat denatured human red cells used as a control, and lyophilized reconstituted human red cells. All labeled red cells were injected as a 40-50% cell suspension (1 ml volume) in saline. Peripheral blood samples were collected from the tail at 10 minutes and 1, 2, 4, and 8 hours post-infusion. Samples were weighed and counted to determine the amount of blood collected and the amount of 51-Cr activity present. The results are shown in Table 11.

Table 11. Results From Five Heterologous Cell Survival Studies in Tolerant Rats. (Literature Values Included for Observed Cell Survival in Humans)

Cells	% Recovery of Injected Dose	Rat Model Half-Life (Hrs)	Observed Lifetime in Humans (Days) +
Fresh, Non-lyoph.	101.7 ± 7.7 (n=11)	8.0 ± 0.5 (n=11)	120
Fresh		13.3 ± 4.8 * (n=52)	

Table 11 (continued)

Cells	Injected Dose	Half-Life	Lifetime in Humans +
Fresh		11.0 ** (n=4)	
Sickle Cells *	57.0 ± 18.3 (n=27)	3.5 ± 1.4 (n=27)	30-40
Stomatocytes **	55 (n=4)	1.0 (n=4)	9
Lyophilized	79.4 ± 5.2 (n=17)	3.5 ± 0.1 (n=17)	
Heat Denatured	63.3 ± 12.5 (n=3)	0.9 ± 0.2 (n=3)	

* Sickle cell data from Castro et al. (14).

** Stomatocyte data from Mentzer et al. (15).

+ Human red cell lifetime data are literature values (14, 15).

All other data in Table 11 are Cryopharm values.

The values reported for sickle and stomatocytic cells indicate that the observed ratio of half-lives in the rat model compared to control cells are on the same order of magnitude as the observed circulation lifetimes for the cells in humans. For example, the ratio of the stomatocyte circulation half-life to control cells in rats is 1:11 hours, while the observed ratio of stomatocyte:control lifetime in humans is 9:120 days, or about 1:13 (see Mentzer et al.). The same holds true for sickle cells, in which the ratio of half-lives for sickle cells versus control cells in rats is 1:4, while the ratio of sickle:control circulation lifetime in humans ranges from 1:7 to 1:3 (see reference 16).

In these studies the use of the single isotope technique may overestimate the survival of the red cells, especially if rapid destruction of red cells occurs (17). In order to address this issue, we compared the experimental time zero CPM/gm RBC to the theoretical time zero CPM/gm RBC, and express this ratio as the Percent Recovery of Injected Dose in Table 11. We used the literature value of 0.0498 ml per 100 gm body weight to estimate the blood volume of each rat (18). Although we cannot accurately know the total blood volume and red cell mass, the Percent Recovery of Injected Dose does indicate the relative confidence that can be placed in the single isotope method in each experiment. As shown in Table 11, the recovery of 101.7% ± 7.7% (experimental time zero value over theoretical) for fresh red cells suggests little early removal of injected

cells. The lower Percent Recoveries exhibited by denatured and lyophilized cells suggests rapid removal, especially in the case of denatured cells. We interpret the biphasic kinetics in the rats receiving denatured red cells to indicate rapid intravascular lysis and removal of the heat damaged cells. The prolonged second phase is likely due to circulating cell-free hemoglobin or free chromium.

While these results must be interpreted cautiously, we believe that the lyophilized reconstituted cells are capable of a significant degree of circulation. We can compare the half-lives of lyophilized human red cells in tolerant rats with reported comparisons between survival of normal, sickle cell, and stomatocytic cells in the heterologous and autologous systems (see references 14, 15). This issue must now be addressed using autologous studies in normal human volunteers. Our clinical study plan is further discussed under Future Plans. Although the heterologous model is not ideal, it can serve to reveal significant improvements or impairments in cell survival following new process treatments.

FUTURE PLANS

During the upcoming research period (leading to the next scheduled research report due in November 1990), Cryopharm will focus on its first clinical research study. As outlined in the clinical protocol submitted earlier, our goal will be to establish an initial baseline of cell survival. This baseline is vital as we have amassed considerable in vitro information on the properties of lyophilized human red cells, and have established a comparable baseline using a heterologous rat model, and this information must now be evaluated in light of cell circulation.

We propose to use the standard 51-Cr single isotope method (19) to establish our initial baseline in vivo. This method can overestimate cell survival in cases where rapid removal of infused, labeled cells occurs (17, 19). Beutler and West (17) have compared the 51-Cr and 91-Tc labeling methods, and conclude that 51-Cr overestimation is limited to about 4% in the range of 10-80% cell viability, with the greatest error occurring when viability is very low. Our lyophilized reconstituted human red cells exhibit over 80% in vitro stability when incubated in autologous plasma at 37 degrees for extended periods in a "pseudo" autohemolysis test. In addition, our experiments using the heterologous rat model did not reveal rapid (i.e., within the first 5-10 minutes post-infusion) clearance of a significant fraction of lyophilized human red cells (this can be compared to the rapid clearance observed with heat denatured control cells). From these results we believe that autologous lyophilized human cells will have sufficient viability, especially within the first 5-20 minutes post-infusion, to permit a reasonably accurate back-extrapolation to time zero.

Our research strategy will be to use the simple and expedient 51-Cr method to establish an initial circulation baseline, and then to evaluate subsequent process modifications that may improve upon this baseline value. We plan to study several new buffer formulations now in development, including use of antioxidants to minimize damage to the red cell hemoglobin and membrane. Our goal will be to significantly improve upon our current 35% yield of intact cells following washing, and to improve upon our initial circulation baseline. We will also explore minimal washing protocols designed to replace the current 3 wash steps with a single wash following reconstitution.

The use of more accurate cell survival determinations, involving 51-Cr and 91m-Tc double labeling (19), can be incorporated into our clinical research program once the fundamental issues of cell yield and viability have been addressed.

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APPENDIX I

Photographs of Current Clean Room Processing.

Figure 1. Class 100 Buffer Preparation Facility.

Cryopharm's Class 100 clean room facility has two connected rooms, one for preparation of sterile lyophilization and rehydration fluids, and one for the actual freeze-drying and packaging of human red cells. This photograph shows the buffer preparation area, which contains a wall-mounted steam sterilizer, storage cabinet for sterile glassware, and a blood bank refrigerator for incoming liquid blood.

Figure 2. Class 100 Lyophilization Facility.

The Class 100 lyophilization room has two flush wall-mounted shelf freeze-dryers. Each freeze-dryer has a stainless steel mounting flange and a central plexiglass chamber door. Liquid units of red cells in lyophilization buffer are placed on the chamber shelves, frozen, and then dried by sublimation under high vacuum. Once the drying cycle is completed, dry nitrogen gas is used to backfill the chamber prior to releasing vacuum to remove the unit. The nitrogen gas passes through a 0.2 micron sterile filter cartridge (shown attached in-line above the chamber door) prior to entering the chamber.

FIGURE 1.

FIGURE 2.

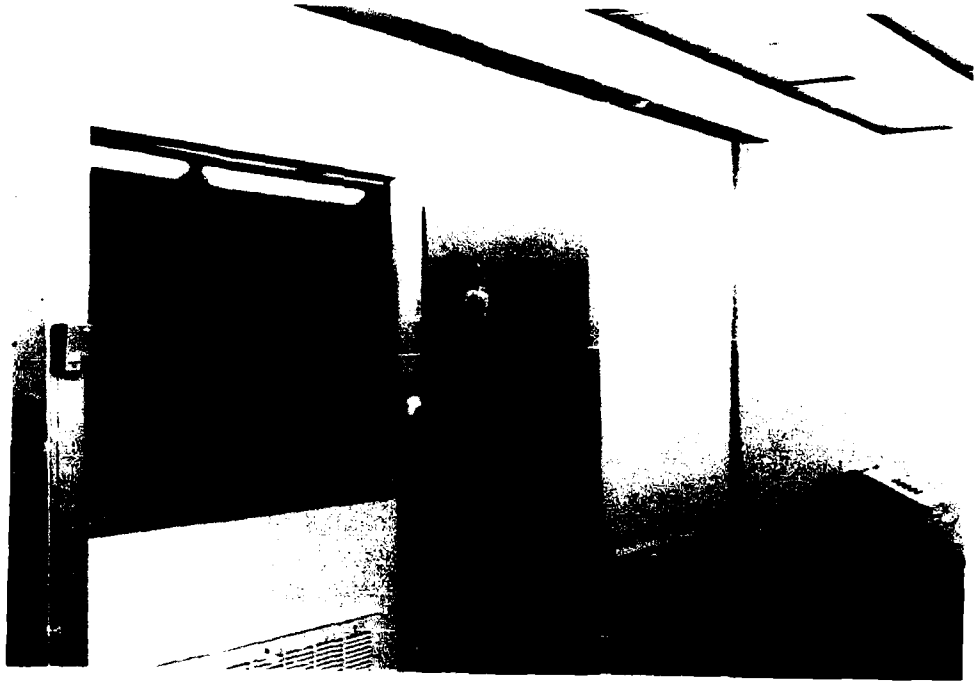


Figure 3. Outer Shell Area Showing Lyophilizer Units and Control Center.

In this Figure the outside shell area housing the clean room and heavy equipment is shown. Each lyophilizer, with its vacuum pumps, condensers, and computer control station, sits outside of the Class 100 room for easy access. Entry to the Class 100 room is via a separate gowning room (entry door at far end in this photograph).

Figure 4. Factory Prepared Sterile Lyophilization Bag.

Cryopharm's first working lyophilization bag prototype is shown as it is delivered from the manufacturer. The thermoformed plastic bag is machine tooled, leak tested, sterilized by exposure to ethylene oxide (EtO) gas, and prepackaged in a sterile wrapper. Sterile docking ports, lines, and spikes compatible with standard blood transfer packs and cell washers are an integral part of the container and permit easy access to the dried contents in a non-sterile environment once the container is sealed.

FIGURE 3.

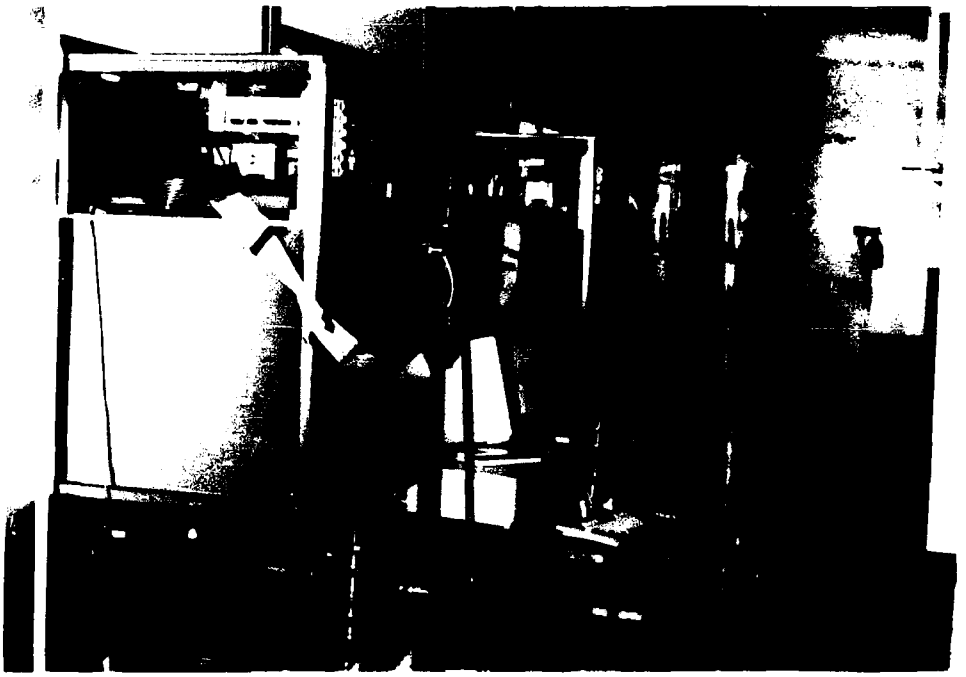


FIGURE 4.

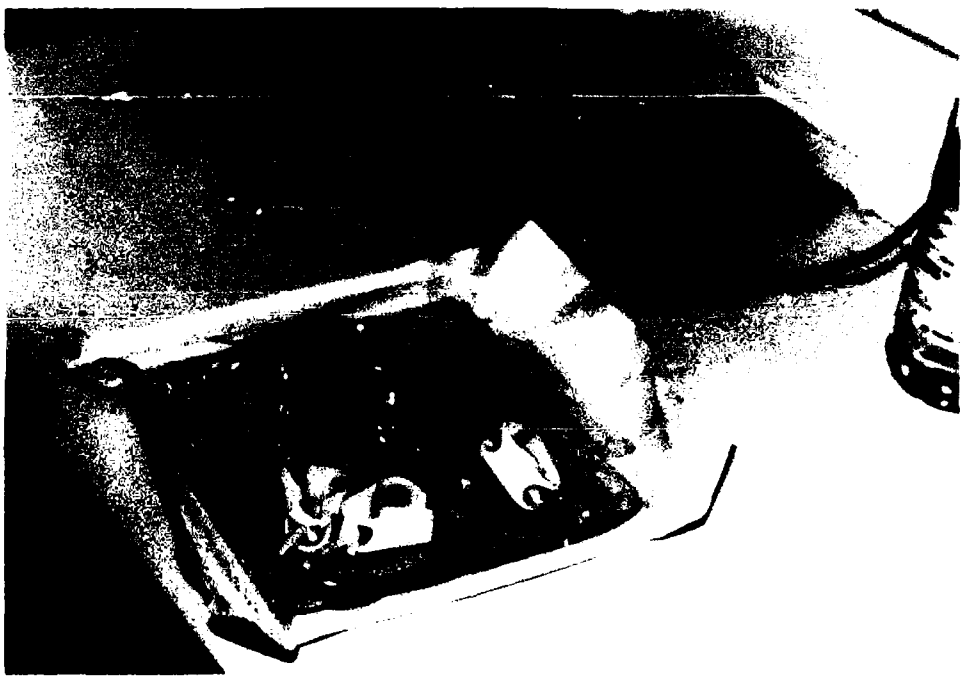


Figure 5. Clean Room Operations in Progress.

This photograph illustrates standard clean room operations in progress. At this point a unit of human red cells has completed its drying cycle and is being removed from one of the shelf lyophilizers. Personnel working in this room observe standard Class 100 working conditions, including use of sterile gowns, hoods, masks, and gloves. Goggles are worn over the eyes and exposed facial areas. This room and the lyophilizer chambers are sanitized on a regular schedule and monitored for microbials by standard "touch plates" or wipe tests and culturing.

Figure 6. Sealing of a Dried Unit Prior to Removal From the Sterile Room.

The dried unit must be sealed at the port which is left open during the freeze-drying process for removal of water vapor by sublimation. The seal is achieved using a 1 kilovolt radiofrequency bag sealer (the welding unit is connected to a transformer outside of the Class 100 room).

FIGURE 5.

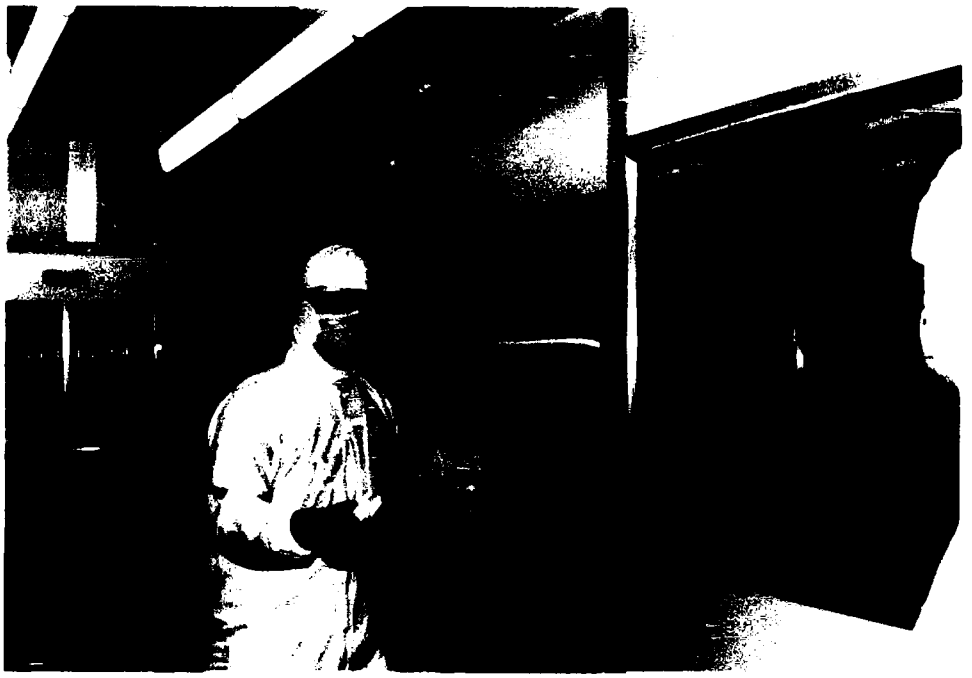


FIGURE 6.

Figure 7. A Sealed Unit of Lyophilized Human Red Blood Cells.

The sealed container is shown with its dried contents of human red blood cells. At this point the air-tight container can be removed from the sterile Class 100 facility and all subsequent manipulations (including air transport, rehydration, washes, and infusion) can be achieved in normal room environments. Note the full red color of the dried red cells, indicative of the high level of oxyhemoglobin preservation in these samples. The unit shown contains one full unit of packed red blood cells.

Figure 8. Reconstitution and Washing of Dried Red Cells.

A dried unit of red cells is rehydrated by adding sterile buffer prepared in Cryopharm's Class 100 buffer preparation room. The buffers are packaged into standard blood transfer bags in the clean room. Use of sterile connectors allows the fluid to be drained into the lyophilization bag in a normal room environment. The rehydrated red cells can then be drained, as shown in this photograph, into a Cobe 2991 cell washing unit. Sterile wash solutions are used automatically from the reservoir bags. We currently use three washes to produce our final cells for infusion. Future research will aim at reducing the number of washes.

FIGURE 7.

FIGURE 8.

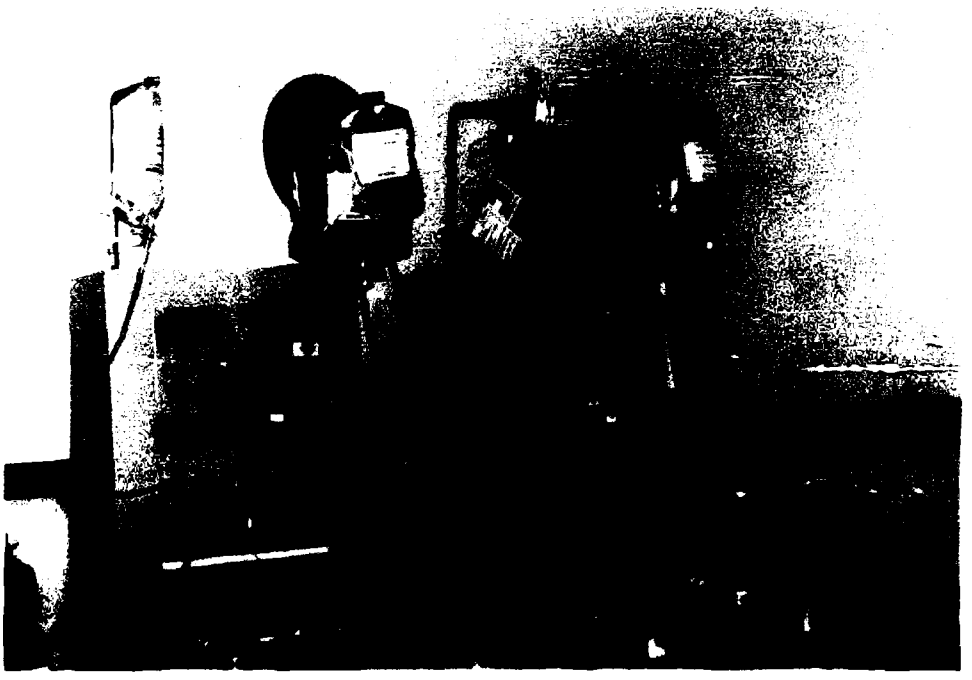
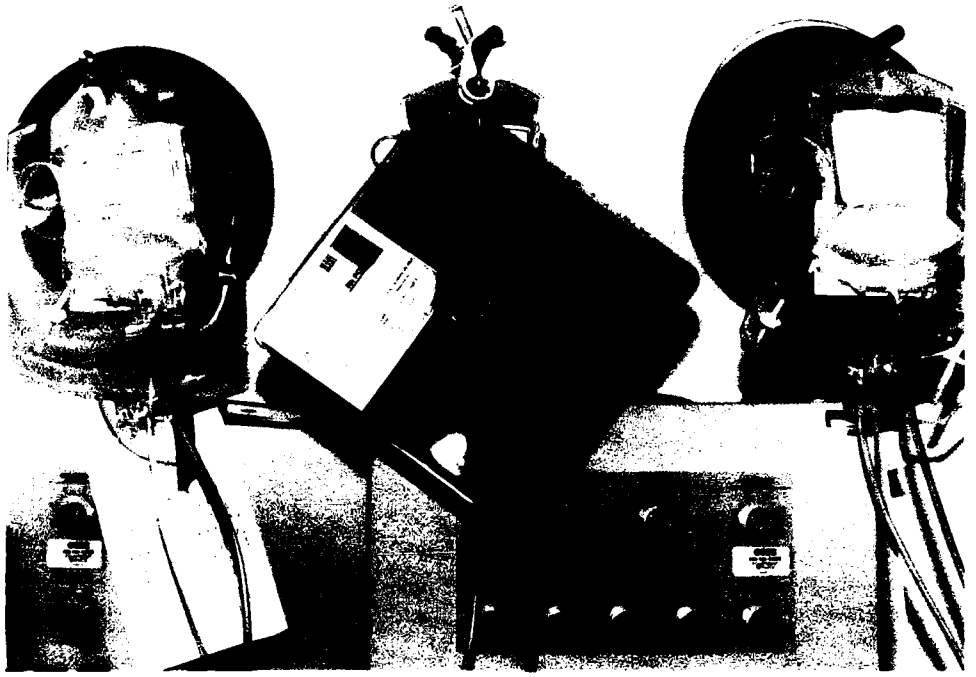


Figure 9. Close-up of Rehydrated Lyophilized Human Red Blood Cells.

This photograph shows a close-up of the ongoing wash procedure from Figure 8. Note the dark red color of the rehydrated suspension of red cells, indicative of the high level of oxyhemoglobin preservation achieved throughout our procedure. This particular unit was from an O-positive donor. Our research has shown that lyophilized reconstituted cells retain their original ABO/Rh grouping, as with refrigerated or frozen red cells.

FIGURE 9.



APPENDIX II. Research Milestones Chart From Cryopharm's September 1989 Research Proposal.

CRYOPHARM CORPORATION
RESEARCH MILESTONES CHART
FREEZE-DRIED RED CELLS

Project Activities	Current Status	Milestone	Projected Start	Projected Completion
Define Shelf Lyophilization Parameters: Define optimal temperature, pressure conditions. Evaluate sample configuration.	No defined cycle	Defined cycle worked-out	Year 1	Year 1
Evaluate Existing Reconstitution Protocol: Mixing and temperature conditions.	~70% initial yield	>80% initial yield	Year 1	Year 1
Optimize Product Properties:				
Cell yield (at infusion stage).	~35-40%	>50%	Year 1	Year 2
Residual moisture (in dry state).	~3%	~1%	Year 1	Year 2
Final product sterility (at infusion stage).	Not done	Demonstrated	Year 1	Year 2
Shelf Life:				
Refrigerated storage.	>10 months	>2 years	Year 1	Year 3
Room temperature storage.	~2 weeks	1-2 months	Year 1	Year 3
Evaluation of Enzyme Converted Red Cells.	Not done	Initial tests	Year 2	Year 2
In vivo Animal Circulation Studies:				
Pilot studies in domestic pigs.	Not done	Done	Year 1	Year 1
GLP quality studies in domestic pigs.	Not done	If pilot tests successful.	Year 2	Year 2
In vitro Animal Red Cell Studies: (Survey models if pig cells do not circulate)	Preliminary data in.	More samples for FDA.	Year 2	Year 2
Plastic Container Development.	First prototype	Developed.	Year 1	Year 1
Streamline Reconstitution and Washes.	Not done	Underway	Year 3	To be deter.
Phase I Clinical Trials of Lyophilized Cells.	Not done	File IND	Year 3	Continues...